

G4 amylase produced by Mariana isolate MS300

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We dived into Challenger Deep at the depth of 10,897 m on March 2 1996 and have collected the mud samples by using 10,000 m class deep sea ROV "KAIKO" (Dive 22). We have isolated strain MS300 which produces exo-maltotetrahydrolase (amylase300) from the mud sample. The amylase300 predominantly maltotetraose from starch. This amylase300 to be very unique. Because, the exo-maltotetrahydrolase of *Pseudomonas stutzeri* strains had been reported^{1,2}), but this enzyme produces glucose maltose and maltotriose besides maltotetraose from starch. We can expect the deep sea environment as the resource of novel microbial biocatalysts.

Keywords : Mariana trench, Challenger deep, enzyme producer, G4 amylase

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1. INTRODUCTION

There are various environments on the earth. Some bacteria adapt its surroundings and produce useful enzymes such as amylases, proteases, cellulases and so on. Most of microorganisms have been isolated from the continents that occupy only 30% of the earth. The ocean occupies 70% of the earth. Our Deep-sea Microorganism Research Group, expects that the novel microorganisms will be isolated from mud of the ocean. Deep-sea is one of ocean environments. Deep-sea has the different ecosystem from the land, because of no sunlight, low temperature and high hydrostatic pressure. So, we expect that Deep-sea is the resource of novel enzyme producer. We had a chance to collect six deep sea mud samples from the Challenger Deep of Mariana trench (which is the deepest point in the world. The mud samples were diluted with Marine broth and spread on various media and isolated various enzyme producer from the mud samples. Our interest was focused on amylase. The amylase has several hydrolysis profiles on starch and is one of the useful enzymes in industrial fields. In this study, we isolated strain MS300 which made the largest halo around the colony among the amylase producers and investigated the property of the amylase produced by MS300.

2. MATERIALS AND METHODS

2.1 Sampling of the sea mud from the Challenger Deep of the MARIANA trench

We dived into the Challenger Deep at the depth of 10,897 m (11°21.111'N, 142°25.949'E) and have collected six mud samples from the bottom. We used 10,000 m class deep sea ROV "KAIKO" to collect the sea mud (Dive 22). The mud samples have been collected by using sterilized mud sampler (ike-ike sampler). The manipulator grasps the sampler and scoops the mud samples. We can get the mud sample which is not contaminated with any other bacteria in the sea.

2.2 Isolation of microorganisms from the sea mud

The mud samples were diluted with Marine Broth 2216 (DIFCO lab) and spread on Marine Broth 2216 (abbreviated MB) plate or MB plate containing 12% NaCl, 1% starch or casein. These media were incubated at 4, 25

or 55°C under 100 or 0.1 MPa. The amylase producer was detected by the halo forming around the colony on the MB containing 1% starch. All isolations have been stored in liquid nitrogen.

2.3 Purification of the amylase (amylase300) produced by strain MS300

The MS300 was aerobically incubated in the MB containing 2% potato starch for 2 days at 25°C. After centrifugation of culture, ammonium sulfate fractionation at 40% of saturation was done to the culture supernatant at 4°C for 16 h. Then, the participation was dialyzed against 50 mM Tris-HCl buffer pH 8.5 at 4°C and applied to a column of DEAE-Toyopeal. The amylase300 was eluted with the gradient of 0 to 0.1 M NaCl gradient. The activity of amylase300 was measured by the method of the *p*-dinitrosalicylic acid reaction³⁾. The purity of each step fraction was checked by SDS-PAGE.

2.4 Thin layer chromatography of amylase300 product

After the amylase300 was reacted with 0.2% potato starch solution, 1 µl of the solution was spotted on 0.2 mm silica gel 60 plate (Merck) and developed with 1-butanol: methanol: water (4: 2: 1). The mixture solution of 0.1% glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose was used as the standard. The oligosaccharide was detected by spraying 1% diphenylamine dissolved in acetone containing 10% phosphoric acid to TLC plate, then heated that at 160°C for 10 min.

3. RESULTS

3.1 The microorganisms isolated from the mud of the Challenger Deep

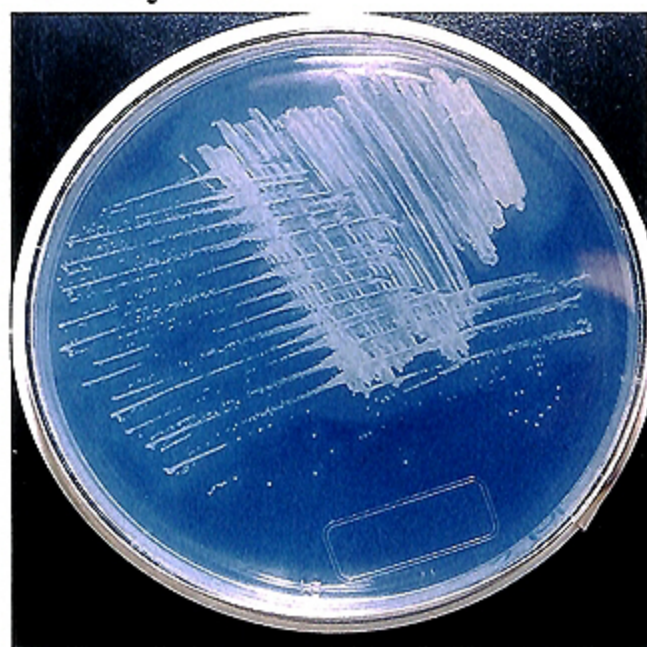
We isolated the various microorganisms by using conventional isolation techniques from the sea mud. The amount of non-extremophiles was $2.2 \times 10^4 - 2.3 \times 10^5$ colonies per gram of dry sea mud (/g of DSM) and about 1% of the isolates from the land. The frequencies of the alkaliphiles and the thermophiles were about 0.67% and 1.7%, almost same as that of the isolates from the land⁴⁾. The barophiles were isolated at the amount of 5-10 colonies /g of DSM. The amylase or protease producers

were isolated. The numbers of the amylase producer was 1.0×10^3 and that of protease producer was 6.0×10^2 colonies /g of DSM. But its frequencies were lower than that of the lands. The halophile or acidophile could not be isolated.

3.2 The isolation of MS300 and purification of its amylase

Among the all amylase producer, we selected strain MS300 which formed the largest halo on the Marine broth

Colony form



Electronmicroscopy

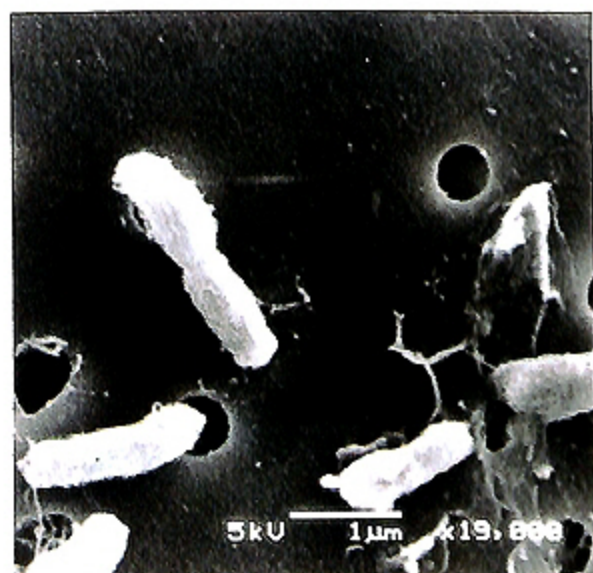


Fig. 1 Colony form and electronmicroscopy (SEM) of strain MS300. MS300 was grown in the Marine broth2216 containing 0.8% potato starch and 0.2% starch azure.

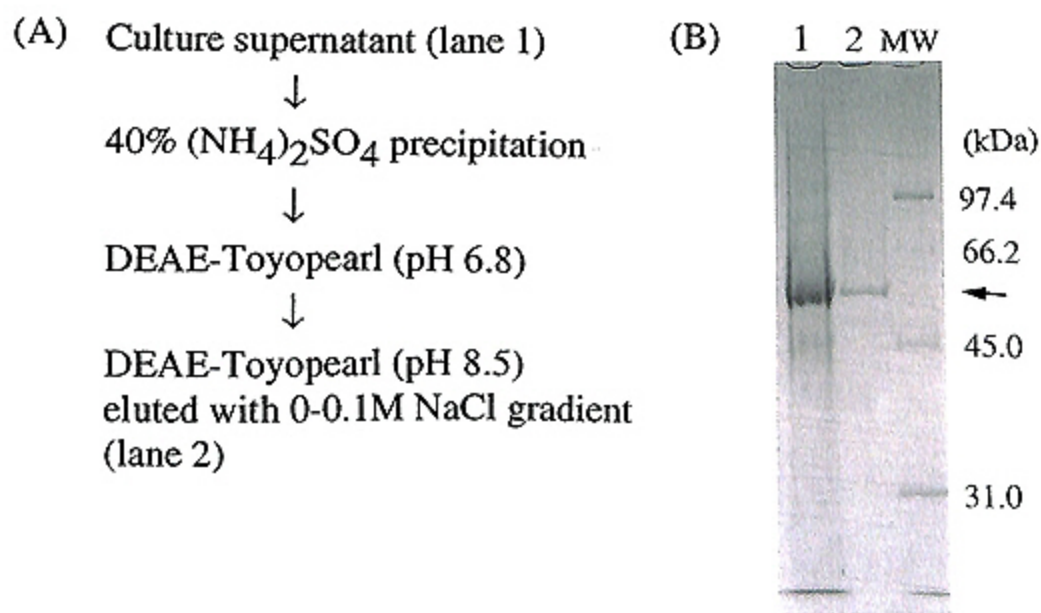


Fig. 2 Purification of amylase300. Panel A shows purification steps of amylase300. Panel B shows SDS-12% polyacrylamide gel electrophoresis of culture supernatant (lane1), purified amylase300 (lane 2) and molecular weight marker (lane MW). Arrow indicates amylase300.

containing 1% starch (Fig.1). The MS300 produced extracellular amylase (abbreviated amylase300). We purified the amylase300 from the culture (Fig. 2). The MS300 was aerobically grown at 25°C in the MB containing 2% starch. Ammonium sulfate fractionation at 40% of saturation was done to the culture supernatant followed by dialysis against 50 mM Tris-HCl buffer pH 8.5 at 4°C and applied to a column of DEAE-Toyopeal. The amylase300 was eluted with the gradient of 0 to 0.1 M NaCl gradient.

The purity of each step fraction was checked by SDS-PAGE. The molecular weight of amylase300 was estimated about 55,000 from SDS-PAGE. This fraction was used further study.

3.3 The reaction profile of amylase300 to the potato starch

The amylase300 produced only maltotetraose from potato starch (Fig. 3). As arrow indicates, the product of

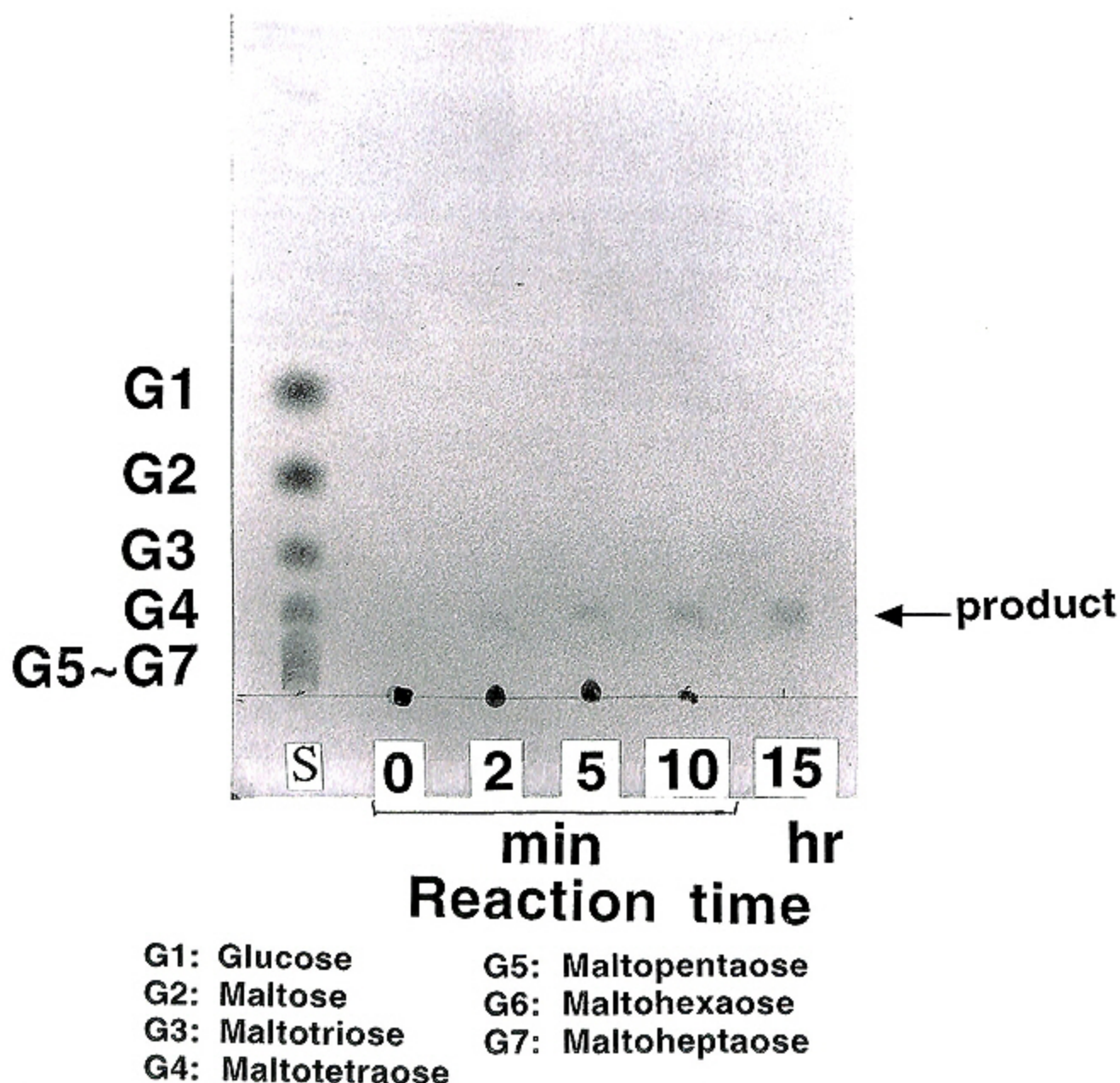


Fig. 3 TLC analysis of enzyme product. TLC analysis was carrying out as written in Materials and Methods. Lane S shows standard of oligosaccharide. Arrow indicates enzyme product.

each reaction time is only one spot. The Rf value of this spot is the same as that of maltotetraose. And, this enzyme could not hydrolyze on α -, β -, γ -cyclodextrin (data not shown). Therefore, we concluded that amylase300 is exo-maltotetraohydrolase.

3.4 The effect of temperature and pH on the activity of the amylase300

Next, we investigated the effects of temperature on the reaction of the amylase300 (Fig. 4). The maximum activity is expressed as 100% in Fig. 4, 5. The activity occurs from 35 to 55°C. The optimum temperature of enzymatic reaction was 40°C. The activity of this enzyme was lost above 60°C. Then, we investigated the effects of pH on the reaction of the amylase300 (Fig. 5). The optimum pH of enzymatic reaction was 6.8.

4. DISCUSSION

In this study, we have first isolated various microorganisms from the mud samples of the Challenger Deep. Some of the microorganisms were the amylase producer. Strain MS300 formed the largest halo around the colony in the amylase producer. The amylase300 which was produced by MS300 was exo-maltotetraohydrolase.

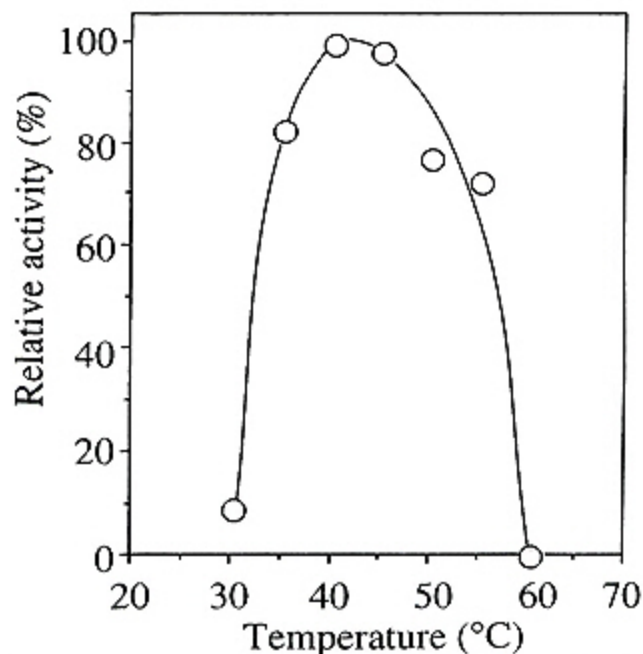


Fig. 4 Effect of temperature on the activity of amylase300. The activity of amylase300 was measured in 0.1 M MOPS-MOPSNa (pH 7.0) by the method described in text. The maximum activity (40°C) is expressed as 100%.

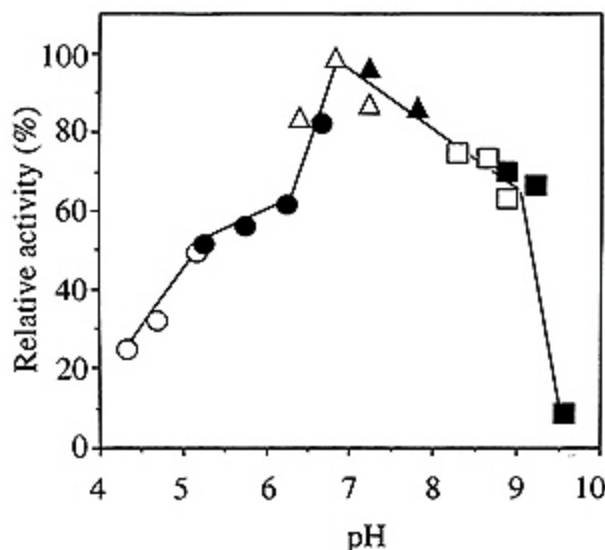


Fig. 5 Effect of pH on the activity of amylase300. The activity of amylase300 was measured as written in the text. Each symbol indicates buffer used in the reaction. Symbols: ○, acetate-sodium acetate; ●, maleic acid monosodium-NaOH; △, Phosphate-NaOH; ▲, MOPS-MOPSNa; □, Tris-HCl; ■, CHES-NaOH buffer. All buffers were used at 0.1M. The maximum activity (pH 6.8) is expressed as 100%.

The exo-maltotetraohydrolase of *Pseudomonas stutzeri* strain had been reported, but this enzyme produces glucose maltose and maltotriose besides maltotetraose from starch. The amylase300 predominantly produced maltotetraose from starch. And the optimum temperature and pH of amylase300 were different from that of *Pseudomonas's* exo-maltotetraohydrolase. So, the amylase300 will be the novel type of the exo-maltotetraohydrolase.

Many microorganisms were isolated from the mud of the Challenger Deep at the depth of about 11,000 m. Furthermore, microorganisms under extreme conditions were also discovered at high frequency. Possibly they have unique properties found in extremophiles.

The deep-sea has been thought that biological activity is very low. But we could isolated many microorganisms. Some of them were enzyme producers, such as amylases proteases. Although we have not fully established isolation and cultivation methods of deep sea microorganisms. We convince that deep sea is one of the new microbial resources. And we can expect the deep sea environment as the resource of novel microbial biocatalysts.

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